

Q<sup>6</sup> probe. A clear band of about 300 by was detected in the fertilized eggs derived from the strains A and C of the transgenic medaka fish. Since the band from the strain C was nearly twice as dense as that from the strain A, it is conceivable that the strain C of the transgenic medaka fish produce the mRNA encoding estrogen receptor in an larger amount than the strain A of the transgenic medaka fish. However, no band was detected in the fertilized eggs derived from the wild medaka fish. From this fact, it is conceivable that the expression amount of the estrogen receptor mRNA is significantly low in the fertilized eggs from the wild medaka fish.--

#### REMARKS

Claims 1-17 are currently pending.

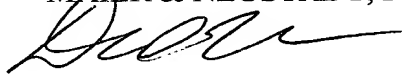
Applicants have now submitted a substitute Sequence Listing and a corresponding computer-readable Sequence Listing. Contents of the paper copy of the substitute Sequence Listing and the computer-readable Sequence Listing are identical. Support for all the sequences listed in the substitute Sequence Listing can be found in the present application. No new matter is introduced by the submission of the substitute Sequence Listing and the computer-readable Sequence Listing.

The specification has been amended for clarity and to give proper citation to the sequences within the substitute Sequence Listing. No new matter is believed to be introduced by the amendments to the specification.

Applicants submit that this application is now in condition for examination on the merits. Early notice to this effect is earnestly solicited.

Respectfully submitted,

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IN THE SPECIFICATION

Page 5, paragraph beginning at line 20 to line 26, delete in its entirety and insert the following.

--The nucleotide sequence of the cloned estrogen receptor cDNA is determined, and the amino acid sequence predicted from the nucleotide sequence is determined. The nucleotide sequence of the medaka-derived estrogen receptor cDNA and the amino acid sequence are shown by Sequence ID No: 1 and Sequence ID No: 2, respectively in the Sequence Listing.

Page 5, paragraph beginning at line 27 to page 6, line 11, delete in its entirety and insert the following.

--In the present invention, the nucleotide sequence for expressing medaka estrogen receptor may have arbitrary length, as long as it comprises at least a coding region (i.e., the nucleotide sequence from 211 to 1935 position represented by Sequence ID No: 1). Furthermore, in the present invention, the amino acid sequence of the medaka estrogen receptor may also have deletion, addition and/or substitution of one or several amino acids in the amino acid sequence represented by Sequence ID No: [1] 2, as long as it has the same function as the protein consisting of the amino acid sequence represented by Sequence ID No: [1] 2.--

Page 16, paragraph beginning at line 25 to page 17, line 20, delete in its entirety and insert the following.

--5. To amplify the medaka estrogen receptor cDNA, a reaction solution (50  $\mu$ L) for PCR was prepared, in accordance with the attached protocol, by using plasmid pMER (10 ng) as a template; 25 pmole of Primer 1 (5'-TCGGTGACATGTACCCTGAA-3') (Sequence ID No: [2] 3) and 25 pmole of Primer 2 (5'-CTGTGTGCTCAGTCTTGAAG-3') (Sequence ID No: [3] 4); and KOD polymerase (TOYOBO) (1  $\mu$ L). PCR was performed by repeating 25 cycles of the following program: 98°C for 15 seconds, 65C for 2 seconds, and 74°C for 30 seconds. After the reaction, the reaction solution containing the PCR product was stored at 4°C. An aliquot (5  $\mu$ L) of the reaction solution containing the PCR product was subjected to electrophoresis on 1% agarose gel. As a result, it was confirmed that the molecular size of the PCR product is about the same as that of a desired product (1.8 kb). From the remaining reaction solution, the amplified DNA fragment was purified by using SUPREC<sup>TM</sup>-02 (TaKaRa) in accordance with the attached protocol. The total amount of the purified DNA fragment was phosphorylated with 2  $\mu$ L of T4 kinase (TOYOBO) in accordance with the attached protocol. After the reaction, the resultant solution was treated at 70°C for 10 minutes.--

Page 18, paragraph beginning at line 24 to page 19, line 24, delete in its entirety and insert the following.

--Half of each caudal fin from the survived adult fish was cut off with scissors. DNA (20  $\mu$ L) was extracted separately from each of the cut caudal fins by using a DNA extraction kit ISOHAIR (WAKO) in accordance with the attached protocol. A reaction solution (100  $\mu$ L) for PCR was prepared, in accordance with the attached protocol, by using the extracted DNA (1  $\mu$ L); two types of primers F1 (5'-CTTCCGTGTGCTCAAACCTCA-3' (Sequence ID No: [4])) 5) and R1 (5'-GTAGGAGGTCATAAAGAGGG-3' (Sequence ID No: [5])) 6) (50

pmole for each); and Ex Taq (TaKaRa Ex Taq RR001B) (1  $\mu$ L). After initial denaturing at 94°C for 2 minutes, PCR was performed by repeating 30 cycles of the following program: 94° for 30 seconds, 60°C for 30 seconds, and 72°C for 90 seconds. Finally, the resultant solution was reacted at 72°C for 6 minutes, and then it was stored at 4°C. An aliquot (10  $\mu$ L) of the PCR solution containing the PCR product was subjected to electrophoresis on to agarose gel. In the case of medaka fish having no chimera gene injected, about 1 kb of DNA band was detected, which was derived from amplification of estrogen receptor gene inherently present in the chromosome of wild medaka fish. In contrast, in the case of medaka fish having a chimera gene injected, a 320 by of DNA band derived from the chimera gene was detected in addition to the above about 1 kb of band. As a result, eight medaka fish with the chimera gene were obtained.--

Page 19, paragraph beginning at line 25 to page 21, line 6, delete in its entirety and insert the following.

--3. Eight medaka fish with the chimera gene were individually crossed with wild medaka fish. From each parent medaka fish, one hundred offspring were raised until they became adult fish. DNA (20  $\mu$ L) was extracted from each caudal fin of these offspring by use of a DNA extraction kit ISOHAIR (WAKO) in accordance with the attached protocol. A reaction solution (100  $\mu$ L) for PCR was prepared, in accordance with the attached protocol, by using the extracted DNA (1  $\mu$ L); two types of primers (5'-CTTCCGTGTGCTCAAACCTCA3') (Sequence ID No: [4] 5) and (5'-GTAGGAGGTCATAAAGAGGG3') (Sequence ID No: [5] 6) (50 pmole for each); and Ex Taq (TaKaRa Ex Tag RR001B) (1  $\mu$ L). After initial denaturing at 94°C for 2 minutes, PCR was performed by repeating 30 cycles of the following program: 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 90 seconds. Finally, the resultant solution was reacted at 72°C

for 6 minutes, and then it was stored at 4°C. An aliquot (10 µL) of the PCR solution containing the PCR product was subjected to electrophoresis on 1% agarose gel. The offspring medaka fish having a 320 bp of DNA band derived from the chimera gene was identified as transgenic medaka fish. As a result, only two of original eight medaka fish actually transferred the chimera gene into their offspring. Therefore, two strains of transgenic medaka fish (designated as strains A and C) were obtained. The number of the transgenic medaka fish obtained herein was small. However, these transgenic medaka fish were crossed with wild medaka fish, and thereby more than about 100 transgenic medaka fish have been maintained for each strain. In both strains of the transgenic medaka fish, about half of offspring obtained by crossing with wild medaka fish have the chimera gene. From this, it was found that either one of two homologous chromosomes had the chimera gene.--

Page 21, paragraph beginning at line 9 to page 22, line 25, delete in its entirety and insert the following.

--The fact that the strains A and C of the transgenic medaka fish produce a mRNA encoding estrogen receptor in a larger amount than wild medaka fish, was demonstrated by the following method. RNA (30 µL) was extracted from about 30 fertilized eggs which were obtained by crossing the transgenic medaka with wild medaka fish and about 30 fertilized eggs which were obtained by mutual mating between wild medaka fish, by use of an RNeasy Mini Kit (QIAGEN) in accordance with the attached protocol. Then, a reaction solution (50 µL) for RT-PCR was prepared by using the extracted RNA (1 µL); three types of primers (50 pmole for each): F1 (Sequence ID No: [4] 5), R1 (Sequence ID No: [5] 6) mentioned above, and R2 (5'-GAGGGACTTTGTTCTTGAC-3') (Sequence ID No: [6] 7); and Ready-To-Go RT-PCR Beads (Amersham Pharmacia Biotech #27-9267-01) in accordance with the attached protocol. After performing initial reactions at 42°C for 30 minutes and 95°C for 5 minutes,

RT-PCR was performed by repeating the 30 cycles of the following program: 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 90 seconds. After completion of the reaction, the reaction solution was stored at 4°C. An aliquot (10 µL) of the RT-PCR solution was subjected to electrophoresis on 1% agarose gel. Thereafter, DNA on the gel was transferred onto a membrane in accordance with the method described in "Molecular cloning - a laboratory manual" (Second edition, J. Sambrook, E. F. Fritsch, T. Maniatis, Cold Spring Harbor Laboratory Press, Pages 9.31-9.62, 1989). Then, Southern Hybridization was performed by using an EcoRI-SalI fragment (354 bp) of the estrogen receptor cDNA as a probe. A clear band of about 300 bp was detected in the fertilized eggs derived from the strains A and C of the transgenic medaka fish. Since the band from the strain C was nearly twice as dense as that from the strain A, it is conceivable that the strain C of the transgenic medaka fish produce the mRNA encoding estrogen receptor in an larger amount than the strain A of the transgenic medaka fish. However, no band was detected in the fertilized eggs derived from the wild medaka fish. From this fact, it is conceivable that the expression amount of the estrogen receptor mRNA is significantly low in the fertilized eggs from the wild medaka fish.--



MARKED-UP COPY

IN THE CLAIMS

Please amend the claims as follows:

6. (Amended) [Transgenic] A transgenic medaka fish [into which the polynucleotide according to claim 1 is introduced] comprising a polynucleotide having a nucleotide sequence of SEQ ID No: 1, wherein said polynucleotide contains a promoter sequence, which is capable of expressing sufficient amounts of a protein encoded by said polynucleotide to produce one or more thrombi.

7. (Amended) [Transgenic] A transgenic medaka fish [into which the polynucleotide according to claim 2 is introduced] comprising a polynucleotide having a nucleotide sequence corresponding to nucleotides 211 to 1935 of SEQ ID No: 1, wherein said polynucleotide is operably linked to a promoter sequence, which is capable of expressing sufficient amounts of a protein encoded by said polynucleotide to produce one or more thrombi.

8. (Amended) A method of producing transgenic medaka fish having one or more thrombi, comprising [the step of] raising the transgenic medaka fish [according to] of claim 6 in the presence of estrogen.

9. (Amended) A method of producing transgenic medaka fish having one or more thrombi, comprising [the step of] raising the transgenic medaka fish [according to] of claim 7 in the presence of estrogen.

10. (Amended) [Medaka] A transgenic medaka fish having one or more thrombi, which is obtained by raising the transgenic medaka fish [according to] of claim 6 in the



presence of estrogen.

11. (Amended) [Medaka] A transgenic medaka fish having one or more thrombi, which is obtained by raising the transgenic medaka fish [according to] of claim 7 in the presence of estrogen.

12. (Amended) A method of testing an estrogen-like [action] activity in test water, comprising [the steps of]:

raising the transgenic medaka fish [according to] of claim 6 in test water; and  
observing whether or not one or more thrombi are formed in the medaka fish after  
[the] said raising [step].

13. (Amended) A method of testing an estrogen-like [action] activity in test water, comprising [the steps of]:

raising the transgenic medaka fish [according to] of claim 7 in test water; and  
observing whether or not one or more thrombi are formed in the medaka fish after  
[the] said raising [step].

14. (Amended) The method according to claim 12, wherein the test water is water taken from the environment.

15. (Amended) The method according to claim 13, wherein the test water is water taken from the environment.

Please add the following new claims:

18. (New) A transgenic medaka fish comprising a polynucleotide having a nucleotide sequence corresponding to nucleotides 211 to 1935 of SEQ ID No: 1, wherein one or more nucleotides are added, deleted, or mutated and the enzyme encoded thereby has estrogen receptor activity, and

wherein said polynucleotide is operably linked to a promoter sequence, which is capable of expressing sufficient amounts of a protein encoded by said polynucleotide to produce one or more thrombi.

19. (New) A method of producing transgenic medaka fish having one or more thrombi, comprising raising the transgenic medaka fish of claim 18 in the presence of estrogen.

20. (New) A transgenic medaka fish having one or more thrombi, which is obtained by raising the transgenic medaka fish of claim 18 in the presence of estrogen.

21. (New) A method of testing an estrogen-like activity in test water, comprising:  
raising the transgenic medaka fish of claim 18 in test water; and  
observing whether or not one or more thrombi are formed in the medaka fish after said raising.

22. (New) The method according to claim 21, wherein the test water is water taken from the environment.

23. (New) The method according to claim 21, wherein the test water is water having a test substance added.

24. (New) A transgenic medaka fish comprising a polynucleotide encoding an amino acid sequence of SEQ ID No: 2, wherein said polynucleotide contains a promoter sequence, which is capable of expressing sufficient amounts of a protein encoded by said polynucleotide to produce one or more thrombi.